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Original Article

Arecoline activates latent transforming growth factor β1 via mitochondrial reactive oxygen species in buccal fibroblasts: Suppression by epigallocatechin-3-gallate

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Areca nut chewing;
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Background/Purpose: Oral submucous fibrosis (OSF) is a premalignant condition caused by the chewing of areca nut (AN). Transforming growth factor β (TGFβ) plays a central role in the pathogenesis of OSF. Connective tissue growth factor (CTGF or CCN2) and early growth response-1 (Egr-1) are important mediators in the fibrotic response to TGFβ in several fibrotic disorders including OSF. Arecoline, a major AN alkaloid, induced the synthesis of CCN2 and Egr-1 in human buccal mucosal fibroblast (BMFs). The aims of this study were to investigate whether arecoline-induced CCN2 and Egr-1 syntheses are mediated through TGFβ1 signaling and to inspect the detailed mechanisms involved.
Methods: Western blot and TGFβ1 Emax® ImmunoAssay were used to measure the effect of arecoline on the TGFβ signaling pathways. 2′,7′-dichlorodihydrofluorescein diacetate and MitoSOX™ Red were used to measure the effect of arecoline on the cellular and mitochondrial reactive oxygen species (ROS).
Results: Arecoline induced latent TGFβ1 activation, Smad2 phosphorylation, and mitochondrial and total cellular ROS in BMFs. TGFβ-neutralizing antibody completely inhibited the arecoline-induced synthesis of CCN2 and Egr-1. Mito-TEMPO, a mitochondria-targeted antioxidant, completely suppressed arecoline-induced latent TGFβ1 activation and mitochondrial and total cellular ROS. Epigallocatechin-3-gallate (EGCG) dose-dependently inhibited arecoline-induced TGFβ1 activation and mitochondrial ROS in BMFs.

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Conclusion: Our results indicated that arecoline-induced mitochondrial ROS plays pivotal roles in the activation of latent TGFβ1 leading to the initiation of TGFβ1 signaling and subsequent increase in the synthesis of CCN2 and Egr-1. EGCG can be a useful agent in the chemoprevention and treatment of OSF.

Introduction

Oral submucous fibrosis (OSF) is a precancerous condition predominantly observed in people of South and Southeast Asian countries.[1–3] The malignant transformation rate of OSF has been reported to be around 7.6% over a 17-year period. More than 5 million people are affected by this disease worldwide. Epidemiological studies have reported that areca nut (*Areca catechu*) chewing is the main etiological factor for OSF. Most patients with OSF present with stiffness of the lip, check, and tongue, leading to varying degrees of limitation of mouth opening and tongue movement. Histopathologically, OSF is characterized by a juxtaepithelial inflammatory reaction followed by progressive fibrosis in the lamina propria and submucosal tissues. OSF is generally considered a collagen metabolic disorder with increased collagen synthesis and reduced collagen degradation. Transforming growth factor β1 (TGFβ1) is the primary growth factor implicated in the development of almost all fibrotic lesions, including OSF. TGFβ1 causes OSF through the activation of myofibroblasts, excessive production of extracellular matrix (ECM), and inhibition of ECM degradation. However, the molecular mechanisms underlying the pathogenesis and progression of OSF are still not thoroughly understood.

The biological activity of TGFβ is restrained by its secretion as a latent complex, which comprises three proteins: bioactive TGFβ, a latency-associated protein (LAP), and a latent TGFβ-binding protein.[4,5] Latent TGFβ-binding proteins interact with ECM proteins and thus bind inactive TGFβ to the ECM. The release of TGFβ from the latent complex, termed as activation, is required for the binding of bioactive TGFβ to its receptors, TGFβ receptor type I and type II, which in turn induce the transcription of target genes through the activation of both canonical (Smad-based) and noncanonical (non-Smad-based) signaling pathways. This process is considered a critical step in the control of TGFβ activity.

Exposure of oral keratinocytes to arecoline, a major areca nut alkaloid, up-regulates αvβ6 integrin expression and promotes TGFβ1 activation through the M(4) muscarinic acetylcholine receptor.[6] Furthermore, the αvβ6-dependent TGFβ1 activation induces the differentiation of oral fibroblasts into myofibroblasts and up-regulates genes associated with tissue fibrosis. In addition, arecoline directly stimulates the production of collagen and differentiation of buccal mucosal fibroblasts (BMFs) into myofibroblast.[7] Moreover, arecoline up-regulates genes associated with ECM formation and remodeling, such as plasminogen activator inhibitor-1, tissue inhibitor of metalloproteinase-1, S100A4, transglutaminase 2, connective tissue growth factor (CTGF or CCN2) and early growth response-1 (Egr-1), in BMFs.[7–13] These genes are induced by TGFβ. However, the effect of arecoline on TGFβ signaling in BMFs has yet to be reported. Deriving a detailed understanding of the molecular mechanisms underlying the arecoline-induced gene expression in human BMFs may provide insights into the development of novel strategies for the treatment of OSF.

Reactive oxygen species (ROS) are formed in substantial amounts in the oral cavity during areca nut chewing.[14] Moreover, arecoline increases intracellular ROS levels in human oral keratinocytes and fibroblasts.[15,16] We previously reported that ROS mediated the arecoline-induced synthesis of CCN2 and Egr-1.[7,10] However, the intracellular sources of ROS involved remain unclear. Identifying the specific redox-sensitive signaling pathway involved in the arecoline-induced synthesis of CCN2 and Egr-1 may facilitate understanding the role of oxidative stress in the pathogenesis of OSF. Furthermore, ROS activates TGFβ from the latent complex.[17] Therefore, this study investigated whether the arecoline-induced synthesis of CCN2 and Egr-1 is mediated through ROS-induced latent TGFβ1 activation and determined the cellular sources of ROS in human BMFs.

Materials and methods

Materials

Arecoline, atropine (a nonselective muscarinic acetylcholine receptors antagonist), N-acetyl-cysteine (NAC, a general antioxidant), allopurinol (a xanthine oxidase inhibitor), polyethylene glycol (PEG)-catalase (a cell-permeable hydrogen peroxide [H_2O_2] scavenger), diphenylene iodonium (DPI, a NADPH oxidase inhibitor), Nω-nitro-L-arginine methyl ester (L-NAME, a nitric oxide synthase inhibitor), mannitol (a hydroxyl radical [$^{\bullet}OH$] scavenger), uric acid (a peroxynitrite anion scavenger), and epigallocatechin-3-gallate (EGCG) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). A matrix metalloproteinase (MMP) inhibitor GM6001, specific TGFβ type I receptor inhibitor SB431542, and specific Smad3 inhibitor SIS3 were obtained from EMD Millipore (Billerica, MA, USA). A TGFβ-neutralizing antibody and mouse IgG1 isotype control were purchased from R&D Systems (Minneapolis, MN, USA). Mito-TEMPO (a specific scavenger of mitochondrial superoxide [$^{\bullet}O_2^-$]), Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP, an antioxidant having superoxide dismutase- and catalase-like activity); and antibodies for CCN2, Egr-1, and β-actin

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